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Complement inhibition attenuates brain edema and neurological deficits induced by thrombin*

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Summary

The present study examined whether thrombin activates the complement cascade in the brain and whether N-acetylheparin, an inhibitor of complement activation, attenuates brain injury induced by thrombin. There were three sets of studies. In the first set, rats had an intracerebral infusion of either five-unit thrombin or a needle insertion. Brains were sampled at 24 hours for Western blot analysis and immuno-histochemistry. In the second set, rats received either five-unit thrombin+saline, five-unit thrombin+25 μg N-acetylheparin or five-unit thrombin+100 μg N-acetylheparin infusion. Brains were sampled 24 hours later for water content measurement. In the third set, rats received either five-unit thrombin+saline or five-unit thrombin+100 μg N-acetylheparin. Behavioral tests sensitive to unilateral striatal damage were carried out for two weeks. Western blotting demonstrated that complement C9 and clusterin levels increase 24 hours after thrombin infusion ($P < 0.01$). Both C9 and clusterin positive cells were found around the injection site. High-dose (100-μg) but not low-dose (25-μg) N-acetylheparin attenuated thrombin-induced brain edema (81.5 ± 0.4% vs. 83.7 ± 0.3% in the vehicle, $P < 0.05$). Behavior was also significantly improved by N-acetylheparin ($P < 0.05$). In conclusion, thrombin-induced edema formation and neurological deficits were both reduced by N-acetylheparin. This suggests that inhibition may be a novel treatment for the thrombin-induced brain injury that occurs in intracerebral hemorrhage.

Keywords: Cerebral hemorrhage; thrombin; complement; N-acetylheparin; brain edema.

Introduction

Mechanisms of edema formation after intracerebral hemorrhage (ICH) have been identified during the past decade [14]. We now know that several processes are responsible for edema formation around the clot. These include hydrostatic pressure during the clot formation, clot retraction, coagulation cascade activation with thrombin production, erythrocyte lysis with hemoglobin induced toxicity, and complement cascade activation in the brain parenchyma [14].

Thrombin, in particular, has been shown to play a major role in early edema formation after ICH. Indeed, a thrombin inhibitor (argatroban) reduced ICH-induced edema formation when given after six hours in a rat ICH model [9] and there is some evidence to support human efficacy [11].

The present study, therefore, examined whether thrombin can activate the complement cascade and whether complement inhibition can reduce thrombin-induced brain injury.

Materials and methods

Animal preparation

The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. Adult male Sprague-Dawley rats (275–325 g, Charles River Laboratories, Portage, Michigan) were anesthetized with pentobarbital (40 mg/kg, i.p.). Aseptic precautions were utilized in all procedures. A polyethylene catheter (PE-50) was then inserted into the right femoral artery in order to monitor arterial blood pressure and to obtain blood samples for analysis of blood gases, blood pH, hematocrit, blood glucose concentration. Body temperature was maintained at 37.5 °C by using a feedback-controlled heating pad.

Intracerebral infusion

Before intracerebral infusion, the rats were positioned in a stereotactic frame (Kopf Instrument, Tujunga, CA), the scalp was incised along the sagittal midline using a sterile technique. A cranial burr hole (1 mm) was drilled near the right coronal suture 4.0 mm lateral to the midline and a 26-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma). Thrombin, saline or throm-

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bin plus N-acetylheparin were infused at 5 μl/min into the right basal ganglia using a microinfusion pump. After infusion, the needle was removed and the skin incisions were closed with sutures. Animals were allowed to recover.

Experiment groups

There were three sets of studies. In the first set, rats had intracerebral infusion of either five-unit thrombin or a needle insertion. Brains were sampled at 24 hours for Western blot analysis and immunohistochemistry. In the second set, rats received either five-unit thrombin+saline, five-unit thrombin+25 μg N-acetylheparin or five-unit thrombin+100 μg N-acetylheparin infusion. Brains were sampled 24 hours later for water content measurement. In the third set, rats received either five-unit thrombin+saline or five-unit thrombin+100 μg N-acetylheparin. Behavioral tests sensitive to unilateral striatal damage were carried out for two weeks.

Brain water content measurement

The rats were sacrificed by decapitation under deep pentobarbital anesthesia (60 mg/kg i.p.). The brains were removed immediately and a 3 mm thick coronal brain slice 4 mm from the frontal pole was cut. That slice was divided into four samples, ipsilateral and contralateral basal ganglia, and ipsilateral and contralateral cortex. Cerebellum was obtained as a control. Tissue samples were weighed on an electronic analytical balance to obtain the wet weight (WW). The tissue was then dried in a gravity oven at 100 °C for more than 24 hours to determine the dry weight (DW). Tissue water contents (%) were calculated as $[(WW - DW)/WW] \times 100$.

Western blot analysis

The rat brains were perfused with saline, and a coronal brain slice was cut as described for brain water content measurements. The brain tissue was immersed in 0.5 ml of Western blot sample buffer and then sonicated for Western blot analysis [15]. Primary antibodies were rabbit anti-complement C9 polyclonal antibody (gift from Dr. P. Morgan, University of Wales) and rabbit anti-clusterin polyclonal antibody (gift from Dr. M. Griswold, Washington State University). The relative densities of complement C9 and clusterin protein bands were analyzed using the NIH image software.

Immuno-histochemistry

Immuno-histochemical studies were performed according to our previous report [15]. Brain sections were incubated according to the avidin-biotin complex technique. Primary antibodies were rabbit anti-complement C9 polyclonal antibody (gift from Dr. P. Morgan, University of Wales) and rabbit anti-clusterin polyclonal antibody (gift from Dr. M. Griswold, Washington State University). Normal rabbit IgG was used as negative control.

Behavioral tests

Animals were placed in a cylindrical enclosure to record preferential use of the non-impaired forelimb for weight shifting movements during spontaneous vertical exploration. The percentage independent use of the non-impaired forelimb (ipsilateral to the injection side), that for the contralateral forelimb, or for both forelimbs together in rapid succession for stepping movements along the walls of the cylinder were calculated. A single score was then used to reflect forelimb use asymmetry: percentage ipsilateral limb use minus percentage contralateral limb use (low score = better function). In addition, a vibrissae-stimulated forelimb placing test (10 trials per side for each rat) was used to examine sensorimotor/proprioceptive capacity (high score = better function) [6]. All behavior was scored by experimenters who were blind to both neurological and treatment conditions. These tests are highly correlated with extent of striatal injury without being influenced by repeated testing.

Statistical analysis

All data in this study are presented as mean ± standard deviation. Data were analyzed with ANOVA using the Scheffe F test or Mann-Whitney U rank test. Significance levels were measured at $P < 0.05$.

Results

Mean arterial pressure, blood pH, arterial oxygen and carbon dioxide tensions, hematocrit, and blood glucose were controlled within normal ranges.

Western blot analysis demonstrated that C9 had a seven-fold increase 24 hours after intracerebral infusion of five-unit thrombin (3329 ± 433 vs 458 ± 395 pixels in saline control, $P < 0.01$). Immuno-histochemistry showed that C9 was deposited on neuronal membranes, indicating activation of the complement cascade and the formation of membrane attack complex. Clusterin, an inhibitor of membrane attack complex formation, also increased after thrombin infusion (4925 ± 686 vs 2453 ± 264 pixels in saline control, $P < 0.01$) and was expressed in neurons.

High-dose (100 μg) but not low-dose (25 μg) N-acetylheparin attenuated thrombin-induced brain edema ($81.5 \pm 0.4\%$ vs $83.7 \pm 0.3\%$ in the vehicle, $P < 0.05$; Fig. 1). Forelimb use asymmetry score and forelimb placing score were also significantly improved by high-dose N-acetylheparin ($P < 0.05$; Fig. 2).

Discussion

In the present study, we demonstrated that thrombin can increase complement C9 and clusterin levels in the brain. Complement inhibition with N-acetylheparin, a heparin congener without anticoagulant properties, reduced thrombin-induced brain edema and neurological deficits.

The complement system is involved in various immune reactions, including cell lysis and the inflammatory response [12]. Complement is normally excluded from the brain parenchyma by the blood-brain barrier (BBB), but entry can occur after ICH as a part of the extravasated blood and later as a result of BBB disruption. There is evidence that the complement cascade is

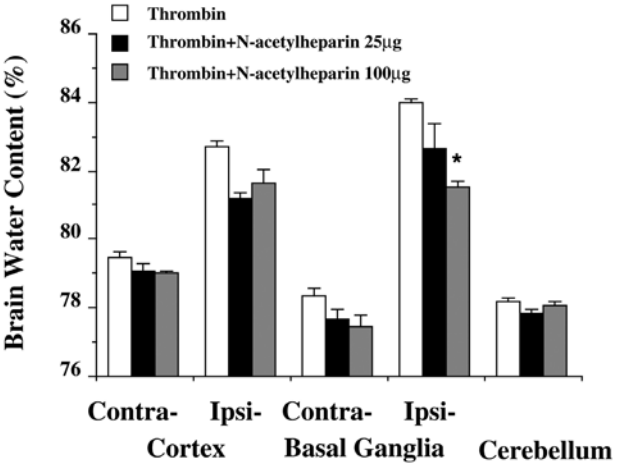

Fig. 1. Brain water content 24 hours after intracerebral infusion of 5 units thrombin, thrombin 5 units + 25 μg N-acetyheparin, and thrombin 5 units + 100 μg N-acetyheparin. Values are mean ± SD, $n = 5$, $^*P < 0.05$ vs. thrombin

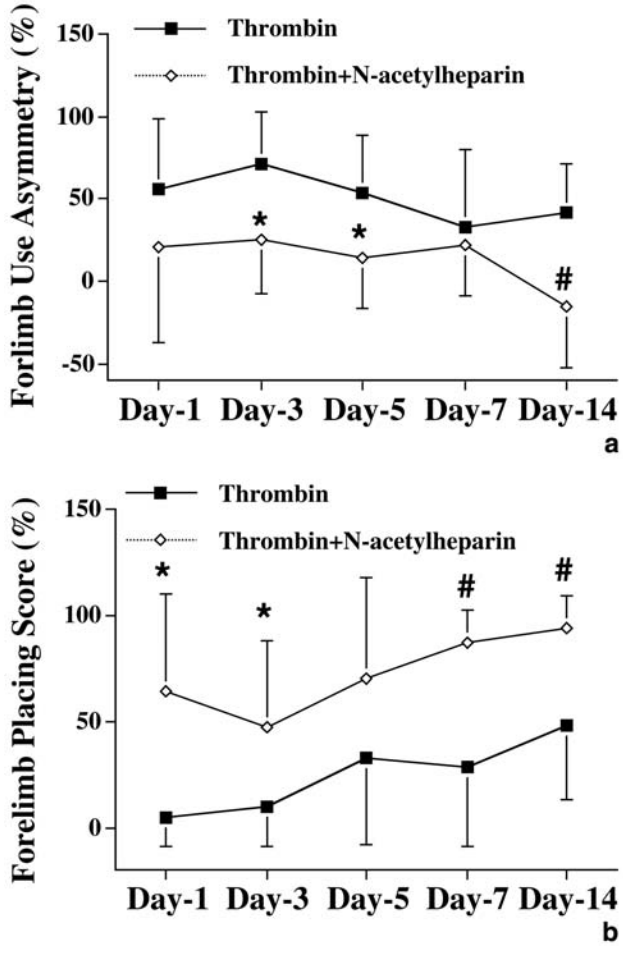

Fig. 2. Forelimb use asymmetry (low score = better function; a) and forelimb placing score (high score = better function; b) at days 1, 3, 5, 7 and 14 after incerebral infusion of 5 units thrombin or 5 units thrombin + 100 μg N-acetyheparin. Values are mean ± SD, $n = 8$, #P < 0.01 and $^*P < 0.05$, Mann-Whitney U rank test

activated in brain parenchyma after ICH. Our previous studies have found that inhibition and depletion of the complement cascade attenuate perihematomal brain edema [7, 13].

The current study indicates that thrombin can activate the complement cascade in the brain. Intracerebral infusion of thrombin resulted in a seven-fold increase in complement C9 and a deposition of complement C9 on neuronal membranes. Complement-related brain injury may be due to membrane attack complex (MAC) formation and the classic inflammatory response. MAC consists of C5b-9 complement forms which are assembled following complement activation [3]. The presence of C9 on cell membranes is an indicator of MAC formation. Its formation causes the production of a pore in the cell membrane that leads to cell lysis. Thus, MAC formation may be involved in the lysis of erythrocytes within the clot after ICH. However, MAC insertion may also occur in neurons, glia and endothelial cells, causing neuronal death and BBB leakage. Our studies have also shown that MAC is assembled after ICH [7]. Recent studies have demonstrated that MAC not only causes cell lysis, but also modulates cellular functions such as the release of cytokines, oxygen radicals, and matrix proteins [5].

Clusterin, an inhibitor of MAC formation, was also up-regulated and found in neurons after intracerebral thrombin infusion. We have also found that it is up-regulated in the brain parenchyma after ICH [7]. The balance between complement activation and clusterin up-regulation may help to determine the extent of brain injury.

The effects of coagulation cascade on complement activation are not well studied. However, several studies suggest that there is a very close relationship between thrombin and complement. About 50% of C3 is cleaved during clot formation [4]. Thrombin can cleave and activate C3 [1]. Thrombin cleaved C3a like fragments are chemotatic for leukocytes and to induce enzyme release from neutrophils [8]. Thrombin can also cleave C5 to produce C5a like fragments which are leukotactic [8].

Although the primary role of thrombin in hemostasis is through cleaving fibrinogen to fibrin and inducing platelet aggregation, other important cellular activities of thrombin may be related to thrombin receptor activation [16]. Three protease-activated receptors (PARs), PAR-1, PAR-3 and PAR-4, have been identified as thrombin receptors [2]. Little is known about

the interaction between the activation of PARs and the complement system. Lidington *et al.* [10] found that thrombin stimulates decay-accelerating factor (DAF) production through PAR-1. However, whether the effects of thrombin on the complement system found in this study are PAR mediated is still uncertain.

In conclusion, thrombin-induced edema formation and neurological deficits were both reduced by N-acetylheparin suggesting that complement inhibition may be a novel treatment for ICH.

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